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Immunological Study on *Salmonellae* Isolated from Different Sources

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ABSTRACT

Salmonella infection is a critical veterinary and medical problem worldwide and is a major issue in the food industry. Non-typhoidal *Salmonella* is known as an important pathogen causing gastroenteritis. The Outer Membrane Proteins (OMPs) of Gram negative bacteria are significant for virulence, host immune responses and drug therapy targets. Enhanced diagnosis of live poultry colonized with *Salmonella* species is required to avoid foodborne diseases. The present study was based on molecular characterization of OMPs among four *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky and *S.* Anatum) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The OMPs profiling showed more than 70 protein bands ranged in size from 208 kDa to below 16 kDa which were detected using Total Lab 1D 12.2 software. All *Salmonella* strains had a band at 54-60 kDa, 45-53 kDa, 36-39 kDa and 26-31 kDa. Eleven strains exhibited a band at 41-46 kDa and 33-35 kDa. Nine strains had a band at 61-69 kDa. Eight strains exhibited a band at 135-145 kDa and 72-79 kDa. Seven strains had a band at 108-123 kDa and 83-91 kDa. In the Western blot analysis, the prepared hyperimmune anti serum of each *Salmonella* serovars reacted with the 35 kDa protein band. It is concluded that the identification of novel immunogenic proteins would be useful in developing ELISA-based diagnostic assays with a higher specificity.

Key words: Outer Membrane Proteins, *Salmonella*, SDS-PAGE, Western blotting.

INTRODUCTION

Salmonellosis is the most commonly reported foodborne zoonotic disease in humans that can cause chronic illness, mortality and societal expenses. The causative agent of salmonellosis includes a variety of *Salmonella enterica* serovars. While more than 2500 serovars of *S. enterica* have been reported, among those *S.* Typhimurium was reported to be second most prevalent serovar of zoonotic significance isolated from humans worldwide. Several countries are confronting this public health crisis due to its resistance to antimicrobial agents and rapid transmission of *Salmonella* via food and water. These organisms are associated with poultry gut, thus the consumption of contaminated poultry meat, egg and contact with infected birds are the main routes of transmission to humans (Prejit et al., 2018). *Salmonella* is categorized according to the different antigens found in the cell wall of bacteria, the O antigen is recognised as somatic antigens and the H antigen is constituted by polymerized subunits of flagellin, while the virulence-associated antigen expressed in the surface of some *Salmonella* strains is known as Vi or K antigen. The use of a number of antisera directed to some of those surface antigens of *Salmonella* constitutes a universal subtyping method called serotyping (Quintana-Ospina et al., 2018). The predominant serotypes present in Egyptian poultry farms are *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis (Sedeik et al., 2019).

Non-typhoidal *Salmonella* is a fundamental cause of food-borne disease globally. It is a universal public health interest, reporting more than 94 million cases and 115,000 deaths every year, with disproportionate influence in developing countries. Salmonellae were revealed in 5% of minced meat samples, 10% of the 20 burger samples, 35% of sausage samples and 25% of poultry products. *Salmonella* isolates were revealed as *S.* Infantis, *S.* Lagos, *S.* Bolombo, *S.* Cerro, *S.* Enteritidis, *S.* Kentucky, *S.* Newlands, *S.* Newport, *S.* Saintpaul, *S.* Sandiego, *S.* Senftenberg and *S.* Typhimurium (EI Jakee et al., 2014).

Another serious health problem that affects antimicrobial treatment is the existence of multidrug-resistant (MDR). Many studies show that infections

produced by MDR strains are more serious than these produced by susceptible strains (Djeghout et al., 2017). Food-borne salmonellosis is a massive public health problem not only in the developing countries but also in industrialized countries, resulting in increasing incidence of enteric diseases, hospitalizations and even deaths every year globally. As one of the most common food borne pathogens, *Salmonella* infects more than 160,000 individuals in the European Union annually, with a morbidity rate of 35 cases per 100,000.6 *Salmonella* was the second etiologic agent which is laboratory confirmed responsible for 229 (30%) recorded outbreaks of food poisoning in the United States and the economic cost of *Salmonella* infections is $2.4 billion annually (Wang et al., 2017). Several diagnostic tests for detecting of *Salmonella* infections in poultry were developed. *S.* Typhimurium (0.6) and *S.* Enteritidis (0.5%). were isolated from eggs (El Jakee et al., 2016).

Cultural isolation is the standard technique for detecting salmonellae in hatcheries and breeding flocks. Cultural procedures for the detection of *Salmonella*, however, are laborious, costly, time-consuming and individual birds intermittently excrete *S. enterica* or may remove the infection completely. Therefore, the designing of dependable screening tests would help identify *Salmonella* presence in hatchery environments and flocks. Serological approaches such as ELISA could help to identify the existence of infected and carrier birds and also silent transmission throughout the flock, that can be missed by traditional bacteriological methods because of the sporadic *Salmonella* shedding (Manoj et al., 2015).

Improvement of detection methods and development of new vaccines would simplify the detection, characterization, and validation of previously unbeknown immunogenic proteins (Meyer et al., 2012). The outer membrane is a continued structure on Gram-negative bacteria surface and has special importance as among the potential protective immunity targets. Recent researches have tended to focus on the Outer Membrane Proteins (OMPs) proposing the presence of *Salmonella* protective immunogenic elements. The OMPs have been identified to be immunogens for evolving active/protective immunity against *Salmonella* and thus, have tremendous possibility to be used in vaccination. OMPs have been inspected as potential candidates for vaccine, virulence factors and those surface exposed proteins play a vital role in pathogenic mechanisms including host cells motility, adhesion and colonization, injection of toxins and cellular proteases, and the formulation of channels for the sweeping of antibiotics (Singh et al., 2017).

The OMPs are effective immunogens on the bacterial surface, which have been used in many trials to check their ability as a vaccine candidate in poultry. Studies have been focused on evolution of OMPs diagnostic antigen. OmpC, OmpF, OmpD are the principal *Salmonella* OMPs (Prejit et al., 2018).

The present study aimed to characterize the OMPs of *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky and *S.* Anatum) collected from different sources and to identify antigenic proteins by Western blotting.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Institutional Animal Care and Use Committee of Cairo University, Giza, Egypt (Vet CU20022020145).

Bacterial strains

Twelve *Salmonella* isolates collected from duckling, chicken, and poultry feed were obtained from the reference laboratory for veterinary quality and control on poultry production (Table 1).

Table1. *Salmonella* strains used in the present study

Groups	Serotype	Antigenic structure			Source of strains
		Somatic (O) antigen	Flagellar (H) antigen		
			Phase1	Phase2	
Group (1)	*S.* Typhimurium	1,4,[5],12	i	1,2	Duckling
	S. Typhimurium				Duckling
	S. Typhimurium				Poultry feed
Group (2)	*S.* Enteritidis	1,9,12	g,m	------	Chicken
	S. Enteritidis				Chicken
	S. Enteritidis				Duckling
Group (3)	*S.* Kentucky	8,20	i	z6	Duckling
	S. Kentucky				Duckling
	S. Kentucky				Chicken
Group (4)	*S.*Anatum	3,{10}{15}{15,34}	e,h	1,6 [z64]	Chicken
	*S.*Anatum				Chicken
	*S.*Anatum				Chicken

Confirmation of the isolates

The collected isolates were tested for purity using xylose lysine deoxycholate (Oxoid). Confirmation of the isolates using biochemical characterization and serological identifications (with agglutination tests with specific O and H antisera, and classified according to the Kauffmann-White-Le Minora scheme) were performed (Quinn et al., 2002).

Real-time PCR

Molecular confirmation of *Salmonella* isolates was done with *Salmonella* specific primers targeting the *invA* gene by real-time PCR. DNA Extraction performed according to the QIAamp DNA mini kit. Specific primers were used and the cycling program was done according to Daum et al. (2002). Master Mix was prepared according to the Quantitect probe Real-time PCR kit. Results were monitored by the Stratagene MX3005P set.

Isolation of outer membrane proteins

The OMPs from *Salmonella* were isolated as described by Verdugo-Rodriguez et al. (1993) with some modifications. Twenty Four hours cultures of bacterial cells in nutrient broth were centrifuged at 1,400xg at 4°C for 10 minutes. The bacterial cell pellet thereafter, resuspended in phosphate-buffered saline (PBS, pH 7.4), and sonicated at a setting of 20kHz or 20,000 cycles/sec (Vi bra Cell sonicator, Sonic & Material Co., Danbury, Connecticut, USA). Sonicated cells were centrifuged at 1,400 x g at 4°C for 10 minutes, and centrifuge the gained supernatant at 100,000 x g at 4°C for 30 minutes and the pellet resuspended in 20 ml of PBS, pH 7.4 containing 20% Triton X-l00 and incubated at 37°C for 20 minutes. The centrifugation step was repeated and the pellet was resuspended in 1 ml of PBS, pH 7.4, and stored at -20°C until use. The protein content was analyzed by the NanoDrop®ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE USA) at 280 nm at reference laboratory for veterinary quality and control on poultry production. It was suitable for performing Electrophoresis.

***Salmonella* species OMP separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis**

Analysis of protein profiles of the *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky, and *S.* Anatum) was done by sodium dodecyl sulphate polyacrylamide gel electrophoresis which was performed on 12% separating and 5% stacking gels using a discontinuous buffer system in a biorad Protein II vertical unit (BioRad, Richmond, CA, USA) as defined by Laemmli (1970). The OMP extracts were solubilized in treatment buffer containing β-Mercaptoethanol. Samples boiled in a water bath for 90 seconds then quickly transferred to ice water. The separation was carried out at a constant current 150V per gel for about 4 hrs. Gels were stained with Coomassie Brilliant blue R-250 staining solution for 4 hrs at room temperature. After staining, the slab gel was immersed in destaining solution repeatedly until the background became clear (about 3 hours). Finally, gel was washed with distilled water. The gel was viewed and photographed under gel documentation. The pictures of gel and marker were loaded on computer program (TollLab) to calculate the molecular weights of peptide bands.

Preparation of hyperimmune sera against *Salmonella* serovars

Hyperimmune antiserum was obtained from 20 chicks (5 chicks for each strain) inoculated IP with 10^{11} formalin killed *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky and *S.* Anatum) solubilized in Auspharm adjuvant as emulsion (0.5 mg/dose) at 19 and 33 days of age, and an oral booster at 47 days of age. Blood was collected after 7 days and serum was prepared and stored at -20^0C according to the protocol of Muir-Wendy et al. (1998).

Western blot

Proteins from culture supernatant gels were blotted on nitrocellulose membranes in 25 mM Tris-HC1, 192 mM glycine buffer, pH 8.3, containing methanol 20% v/v (Neal, 1981). The transfer was influenced by a current of 100 mA overnight in a Bio-Rad Trans blot cell. Free protein sites were saturated by incubation in blocking buffer containing newborn calf serum (Gibco) 10% v/v in phosphate-buffered saline, pH 7-4, Triton X-100 0.2% v/v for 30 min. The nitrocellulose membrane was then incubated in anti-*Salmonella* diluted 1: 20000 PBS1 in 50 in blocking buffer for 1.5 h. After washing three times for 15 minutes each in phosphate-buffered saline, pH 7.4, Triton X-100 0.2% v/v the nitrocellulose membrane was incubated with Rabbit -anti chicken horse reddish peroxidase-conjugated Ab (KPL) 1:5000 (Secondary Ab) The paper was washed afterward and a chromogen substrate containing tetramethylbenzidine was added.

Calculation of molecular weights of the proteins

The relative migration values of the migrated protein fraction were calculated in relation to protein marker by Total Lab 1D 12.2 software.

RESULT AND DISCUSSION

Salmonellae are significant gastrointestinal pathogens that pose a global threat to public health. A total of 12 *Salmonella* strains were included in the study, 5 of them were isolated from duckling, 6 from chicken and one from poultry feed. The isolates were confirmed to be salmonellae using conventional and molecular methods (Figure 1). The outer membrane is a persistent feature on Gram-negative bacteria surface and has particular significance as one of the potential targets for protective immunity. Determination of the protein content plays an important role in bacterial classification, identification, typing, and comparative studies. New searches on OMPs have suggested the presence of *Salmonella* protective immunogenic components (Singh et al., 2017).

The results of SDS-PAGE showed that more than 70 protein bands ranged in size from 208 kDa to below 16 kDa. (Figure 2). All *Salmonella* strains had a band at 54-60 kDa, 45-53 kDa, 36-39 kDa, and 26-31 kDa. Eleven strains had a band at 41-46 kDa and 33-35 kDa. Nine strains had a band at 61-69 kDa. Eight strains had a band at 135-145 kDa and 72-79 kDa. Seven strains had a band at 108-123 kDa and 83-91 kDa.

Protein bands of 78.1, 51.2, 41.5, 37.3, 35.1, 33.9, 30.7, 27.6, 25.4, and 24 kDa were detected in all *Salmonella* serovars and protein bands of 78.1, 51.2, and 41.5 kDa appeared as major bands in all strains (Aksakal, 2010*)*. The intense protein region which occupied the range from14 and 45 kDa constituted the *Salmonella* specific OMP bands, the higher molecular weight region (higher than 45 kDa) and at the lower molecular weight region (lower than 14 kDa) were bands related or associated to the OMP or residues of flagella and pilus protein (lower than 20 kDa) as recorded by Maripandi and Al-Salamah (2010).

All *S*. Typhimurium had a band at 96-84, 75-72, 69-63, 47-45, 43-42, 37-36, 34-33, 27-26 and 22-20 kDa. The majority of *S*. Typhimurium isolates (74.3%) contained two OMPs of 30.6 and 34.6 kDa, 6 isolates (17.1%) carried three OMPs of 27.2, 30.6 and 34.6 kDa and three isolates (8.6%) contained only a 30.6 kDa (Maripandi and Al-Salamah, 2010). More than 21 OMP bands could be resolved from *Salmonella* Typhimurium, *Salmonella* Breanderp and *Salmonella* Lomita ranging in size from 61.0 kDa to 7.5 kDa **(**Osman and Marouf, 2014).

Among *S*. Enteritidis all strains had a band at 91-82, 72-67, 59-55, 45-43, 39-38, 37-35, 25-29, and 16 kDa. When the protein profiles of *S*. Enteritidis originating from chickens and turkeys were compared, no differences were found among the isolates within this serovar (Aksakal, 2010). *S.* Enteritidis with different OMPs bands were exhibited with a molecular weight ranged from 5-90 kDa and the major OMPs profiles of all *S.* Enteritidis isolates were homogenous with different expression in intensity of protein was observed by Maripandi and Al-Salamah (2010). The whole cell proteins of *S*. Typhimurium and *S*. Enteritidis showed similarity in analysis by SDS PAGE analysis, both strains yielded major bands at 71.4, 67.7, 44.0, and 30.3 kDa (Aksakal, 2010).

In the present study, all *S.* Anatum strains had a band 62-56, 54-52, 46-42, 41-40, 38-37, 35-33, 29-26, and 18-16 kDa. And all *S*. Kentucky strains had a band at 62-60, 54-51, 44-42, 38-37and 29-28 kDa. *Salmonella* Kentucky is among the most frequently isolated *S. enterica* serovars from food animals in the United States (Haley et al., 2019).

Infection with *Salmonella* is a significant medical and veterinary problem globally causing major concern in the food industry. This study implemented the Western blot technique to detect the presence of antigenic proteins of *Salmonella* strains. The result showed that hyperimmune antiserum of each *Salmonella* serovar reacted with the OMP 35 kDa protein band (Figure 3). Another study carried out Western blot analysis against OMP of *S.* Enteritidis, serum antibodies from chicken infected with *S*. Enteritidis reacted with protein band at molecular weight 14.4 and 24 kDa, while antibodies raised against *S*. Typhimurium reacted with protein bands at molecular weights of 17, 24 and 31 kDa (Maripandi and Al-Salamah, 2010). They recorded that 14.4 and 24 kDa proteins were immune response protein and can use for vaccine development.

The finding of the present study highlighted that 35 kDa OMP of *Salmonella* serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Kentucky and *S*. Anatum) is an immune-response protein. This protein can be used for vaccine preparation in future.

Jaradat and Zawistowski (1998) demonstrated the 35 kDa OMP contained an antigen common for all tested *Salmonella* species except atypical species such as *S*. Arizona. The results of the protection studies conducted by El-Tayeb et al. (2019) indicated that the highest protection was observed using the 38 kDa OMP, which provided 100% protection to mice challenged with 50× LD50 of *Salmonella* Typhimurium SA3 and 75% protection to mice subjected to an even higher bacterial challenge of 100× LD50. Therefore, 38 kDa OMP is a promising candidate for the vaccine development against *S.* Typhimurium.

Pandey et al. (2018) concluded that OMP 28 may be proven to be an effective candidate for the development of

recombinant DNA vaccines against salmonellosis. Antigenic bands of *Salmonella* spp. of 10, 15, 17 and 40 kDa and 10, 17, 25, 37 and 75 kDa were detected in 15 out of 18 (83.3 %) and 4 out of 18 (22.2 %) samples from chicken carcasses and egg surface, respectively. Quintana-Ospina et al. (2018) suggested that rOmpC evident by a single protein band of 43 kDa based indirect ELISA as a suitable screening tool for serological monitoring of poultry flocks. Recently, Li et al. (2019) established indirect ELISA using the IpaJ protein (a new antigen reported to be specific to *S.* Pullorum, and not detected in *S.* Gallinarum and *S.* Enteritidis) is a novel method for specific detection of *S.* Pullorum infection, and contribute to eradication of Pullorum disease in the poultry industry.

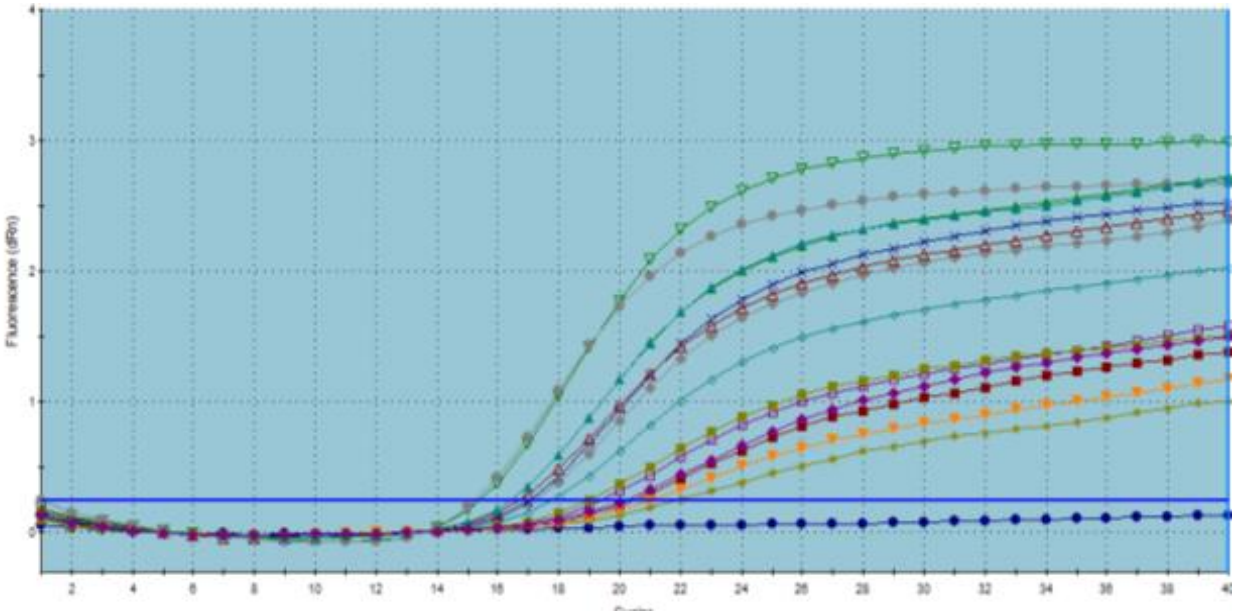

Figure 1.Amplification curves of real time PCR for detection of *invA* gene of studied *Salmonella* serovars by Stratagene MX3005P.

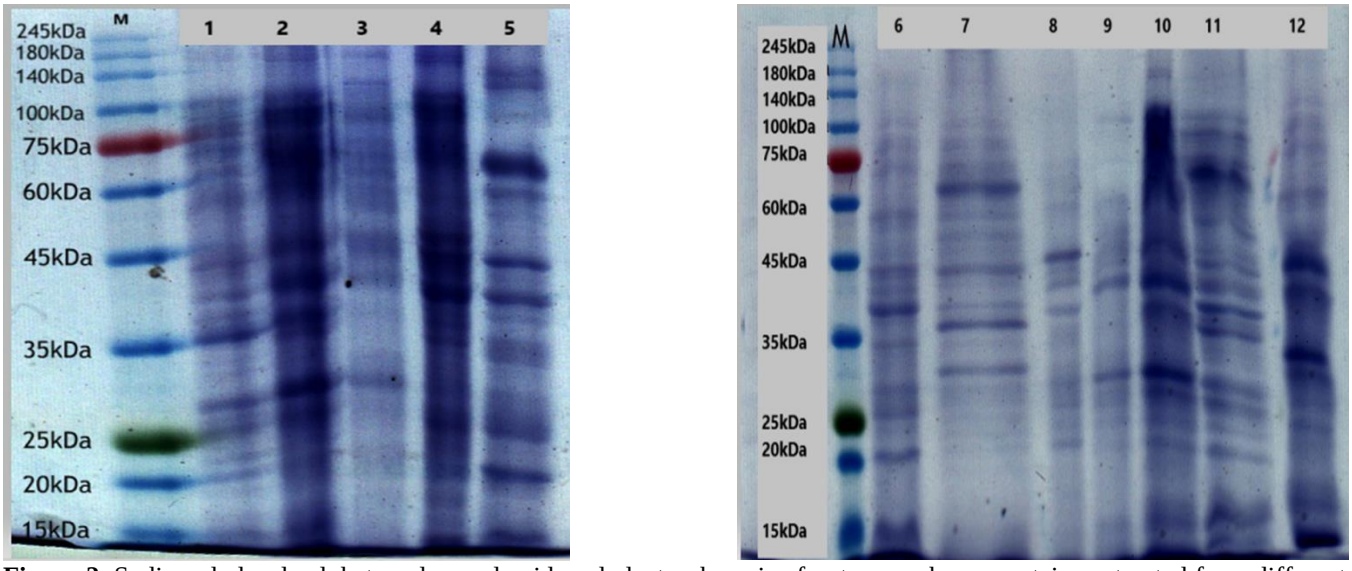

Figure 2. Sodium dodecyl sulphate poly acrylamide gel electrophoresis of outer membrane proteins extracted from different *Salmonella* strains and stained with Coomassie Brilliant Blue R-250. A): Lanes 1, 2 and 3: *S.* Typhimurium, Lanes 4 and 5: *S.* Enteritidis and Lane M: Molecular weight standards. B) Lane 6: *S.* Enteritidis, Lanes 7, 8 and 9: *S.* Kentucky, Lanes 10, 11 and 12: *S.* Anatum and Lane M; Molecular weight standards.

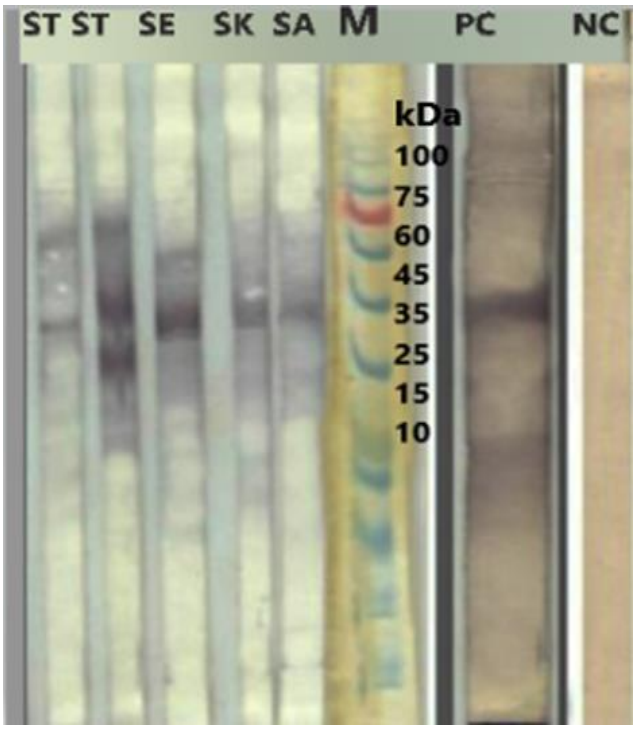

Figure 3. Immunoblot of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins extracted from different *Salmonella* strains. Lane ST: *S*. Typhimurium, Lane SE: *S*. Enteritidis, Lane SK: *S*. Kentuky, Lane SA: *S*. Anatum, Lane M: Molecular weight standards (Marker proteins ranging from 100 kDa to10 kDa), Lane PC: positive control and Lane NC: negative control.

CONCLUSION

It is concluded that *Salmonella* OMP can play an essential role in the induction of immune response in the animals and can be employed as an effective candidate vaccine. Moreover, immunoblotting are helpful for the discovery of antigenic proteins that participate in cross-reactive responses across the different serovars. Further studies are needed to substantiate whether these antigenic proteins are likely to protect against *Salmonella* infection *in vivo*.

DECLARATIONS

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Competing interests

No competing interest exists.

Author's Contributions

All authors contributed equally to this work

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